Clin. exp. Immunol. (1985) **60**, 217–224.

Effect of protein deprivation on immunoregulatory cells in the rat mucosal immune response

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(Accepted for publication 13 November 1984)

SUMMARY

Secretory IgA antibody responses such as those directed against respiratory and enteric pathogens, may be impaired in protein–calorie malnutrition. In previous studies using a rat model of the mucosal immune response to cholera toxin, as little as 2 weeks of severe protein deprivation markedly impaired mucosal anti-toxin production. The present studies examined the effect of protein deprivation on lymphocyte populations which adoptively transfer either priming or suppression of a mucosal anti-toxin response from malnourished donor rats to syngeneic well nourished recipients. Either 2 or 8 weeks of protein deprivation severely impaired the development of thoracic duct lymphocytes which could transfer priming or suppression after intraduodenal priming, and impaired splenic suppressor cell development after s.c. priming. The abrogation of suppression by protein deprivation was dependent on the dose of s.c. antigen used to induce suppression. Refeeding rats after two months of protein deprivation led to recovery of both priming cell and suppressor cell function. Severe protein deprivation induces a reversible defect in both priming and suppressor cell populations; the net effect is an impaired mucosal immune response.

Keywords mucosal immunity secretory IgA malnutrition immunoregulation

INTRODUCTION

The high incidence of intestinal and other mucosal infections in protein–calorie malnourished children may result, at least in part, from defective IgA antibody responses at mucosal surfaces (Chandra, 1975), although other nutritionally related defects, such as impaired epithelial cell integrity, may also be contributory (Koster, 1983). An earlier study from this laboratory showed that as little as 2 weeks of protein deprivation markedly impaired the mucosal IgA antibody response to enteric doses of cholera toxin (CT), an especially efficient mucosal immunogen, in rats (Barry & Pierce, 1979). The mechanism of this effect was not studied in detail, but was shown to involve reduced generation of specific IgA plasmablasts in Peyer's patches and also diminished antigen driven division of sensitized B cells within the intestinal lamina propria.

In recent studies, we have described the appearance and migration of lymphocytes which participate in, or modulate, the mucosal IgA response to CT in rats. These include memory cells and suppressor cells which migrate in thoracic duct lymph after enteric immunization (Pierce & Cray, 1981; Koster & Pierce, unpublished observations), and suppressor T cells which appear in the spleen and other sites after parenteral immunization (Koster & Pierce, 1983). Our purpose in the present study was to determine how these responses are affected by protein deprivation. By transfusing

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thoracic duct lymphocytes or spleen cells from immunized, protein deprived donors into syngeneic, normally fed recipients, we have shown that protein deficiency impairs the development of donor cell populations that mediate either priming or suppression of a specific mucosal IgA response and that this effect can be reversed by refeeding.

MATERIALS AND METHODS

Experimental animals and diets. Seven week old inbred female Fischer rats (CDF [F-344]/CrlBR), weighing 100–120 g, were obtained from Microbiological Associates, Walkersville, Maryland, USA, rested for 3–7 days and distributed into two dietary groups in a conventional rodent colony. One group received a low protein (LP) diet (3·2% protein entirely as casein, Low Protein test diet No. 170580, Teklad, Madison, Wisconsin, USA). The control diet (HP) was identical but contained 24% protein as casein (Taklad Normal Protein Test Diet No. 170590). Although these diets were designed to study only protein deprivation and were otherwise nutritionally complete (Hegsted & Chang, 1965), neither the LP or HP diet contained added zinc, although the casein contained 13–52 parts/10^6 of zinc, and no efforts were made to exclude zinc from the environment. In some instances control groups were fed a standard rat chow, containing 23% protein (NIH Open Formula Rat Ration, Ralston Purina, St Louis, Missouri, USA). All diets were given *ad libitum*. Previous studies have shown that intakes of LP and HP diets were approximately isocaloric with respect to body weight (Bell, Hazell & Price, 1976; Barry & Pierce, 1979).

Immunizations. The preparations of purified CT and formalinized CT (CTd) used are described in detail elsewhere (Pierce, 1978). CT was purchased from Schwarz–Mann Laboratories, Orangeburg, New Jersey, USA; CTd was a gift of Dr R. O. Thomson, Wellcome Research Laboratories, Beckenham, Kent, UK. Each was diluted in sterile 0·01 M phosphate-buffered saline, pH 7·4, containing 0·02% gelatin. For parenteral immunization, CT or CTd were given s.c. in 0·2 ml divided over each flank. For intraduodenal (i.d.) immunization, only CT was used, 0·5 ml being directly injected into the bowel lumen though a small laparotomy (Pierce & Gowans, 1975).

Assay of mucosal anti-toxin response. Anti-toxin containing plasma cells (ACC) were detected in biopsies of jejunum using a highly specific fluorescent antibody technique, described in detail elsewhere (Pierce & Gowans, 1975). The frequency of ACC is expressed as geometric mean frequencies of ACC/mm^3 in the crypt region of jejunal lamina propria. The lower limit of sensitivity in scoring tissue samples (no detectable ACC) was assigned a value of 115/mm^3.

Lymphoid cell preparations. Spleen cells were harvested by dicing the tissue and pressing it through a fine 60 mesh steel screen into RPMI 1640 medium (GIBCO, Madison) with 5% fetal calf serum (FCS) at 4°C. Cells were passed through fine cotton gauze, washed twice with the same medium at 4°C, checked for viability by trypan blue exclusion (always greater than 90%), and resuspended to the desired concentration in RPMI 1640 for i.v. injection. Thoracic duct lymphocytes (TDL) were collected by thoracic duct cannulation (Pierce & Gowans, 1975). TDL from the first 20 h collection, beginning 4 h after surgery, from rats draining more than 10^7 TDL/h, were prepared for i.v. injection and examined for viability as above (always greater than 95%).

Assay for adoptive transfer of mucosal priming or suppression. The adoptive transfer assays have been described in detail elsewhere (Koster & Pierce, 1983; Pierce & Cray, 1981). In brief, donor lymphoid cell populations harvested at varying intervals after s.c. or intraduodenal priming were injected i.v. into syngeneic, non-immune, non-irradiated rats of the same sex weighing 120–140 g. To assay adoptively transferred mucosal priming, the recipients were given 10 μg CT i.d. at the time of cell transfer; jejunal biopsies were taken 5 days later for enumeration of ACC. At this biopsy interval, detectable ACC occur only in rats given IgA memory cells and T helper cells from enterically immunized donors (Pierce & Cray, 1981; Koster, unpublished observations). To assay for adoptively transferred mucosal suppression, recipients were given 10 μg CT i.d. at the time of cell transfer, and again 14 days later; jejunal biopsies for ACC assay were taken 5 days later. Because this immunizing sequence evokes near maximal mucosal ACC responses, a diminished response in cell recipients is considered evidence of transferred suppression (Koster & Pierce, 1983).

Statistics. Statistical analyses used the non-parametric Mann–Whitney U test applied to

geometric mean frequencies of ACC in jejunal lamina propria. *P* values greater than 0·05 are indicated as not significant (NS).

RESULTS

Studies of transferable mucosal priming

Effect of LP feeding on priming transferable by TDL from i.d. immunized donors. TDL from enterically immunized donor rats will adoptively transfer priming for a mucosal anti-CT response to nonimmune recipients (Pierce & Cray, 1981). We sought to determine whether this transferable priming was altered when donors were protein deprived before immunization. Table 1 shows that TDL obtained 11 days after i.d. priming of HP fed donors transferred significant priming for a mucosal anti-CT response to non-immune recipients. In contrast, priming transferred by the same number of TDL from donors fed LP for 2 or 6 weeks before immunization was diminished by 58 and 92%, respectively.

Restoration of transferable priming by refeeding with HP. To determine whether the effect of LP could be reversed by refeeding with HP after enteric immunization, TDL donors were fed LP for 8 weeks, immunized i.d. with 10 μg CT, LP was given for 4 more weeks, then HP was fed for 4 weeks. TDL were collected and transferred to six non-immune recipients (3×10^8 TDL/recipient), 10 μg CT was given i.d., and jejunal ACC were enumerated 5 days later. The mean jejunal ACC response was 2,188 ($\overset{\times}{\div}$1·1)/mm^3, which resembles that previously seen in recipients of 5×10^8 TDL from well nourished donors that were immunized i.d. and fed HP for 8 weeks prior to collection of TDL (2,731 [$\overset{\times}{\div}$1·2]/mm^3; Pierce & Cray, 1981). We conclude that refeeding with HP as late as 4 weeks after enteric immunization restored the ability of subsequently collected TDL to adoptively transfer enteric priming.

Studies of transferable mucosal suppression

Effects of LP feeding on suppression transferred by TDL from i.d. immunized donors. In further studies, we sought to detect suppressor cell activity among immune TDL and determine whether it was also affected by protein deprivation. Transferable suppressor activity in TDL was measured using twice immunized non-malnourished adoptive recipients (see Materials and Methods). Table 2 shows that TDL obtained 15 days after i.d. immunization of HP fed donors suppressed the mucosal anti-CT response in twice immunized recipients by 82% when compared with controls not given immune TDL. In contrast, the same number of TDL from donors fed the LP diet for 2 weeks before immunization were not significantly suppressive in adoptive recipients.

Table 1. Effect of protein deprivation on priming transferred by immune TDL

	TDL donors	TDL recipients		
Diet/duration	Weeks on LP when immunized*	No. TDL transferred	*n*	Jejunal ACC response†
HP	—	None	8	115 (−)
HP	—	1×10^8	8	1860 (1·3)‡
LP/4 weeks	2	1×10^8	10	780 (1·6)‡
LP/8 weeks	6	1×10^8	9	150 (1·3)‡

* Ten micrograms of CT was given i.d. and the indicated diet was continued until TDL were collected 11 days later.

† Non-immune rats were given TDL i.v. and 10 μg CT i.d. ACC were assayed in jejunal biopsies taken 5 days later. Results are expressed as geometric mean ACC/mm^3 ($\overset{\times}{\div}$ s.e.).

‡ Significantly different than result in preceding group, $P < 0{\cdot}001$.

Table 2. Effect of protein deprivation on suppression transferred by immune TDL

Diet/ duration	TDL donors Weeks on diet when immunized*	TDL recipients No. TDL transferred	*n*	Jejunal ACC response†	Percentage suppression‡
HP	—	None	8	11,390 (1·3)	—
HP/4 weeks	2	1×10^8	11	2,090 (1.7)§	82
LP/4 weeks	2	1×10^8	9	9,700 (1·5)¶	15

* Ten micrograms of CT was given i.d. and the indicated diet was continued until TDL were collected 15 days later.

† Non-immune rats were given TDL i.v. and 10 μg CT i.d. The i.d. dose of CT was repeated on day 14 and jejunal biopsies were taken on day 19. Resuls are expressed as geometric mean ACC/mm^3 ($\overset{\times}{\div}$ s.e.).

‡ Compared with responses in rats not given immune TDL.

§ Significantly lower responses than in rats not given immune TDL, $P < 0{\cdot}001$.

¶ Significantly greater than response in preceding group, $P < 0{\cdot}001$; not significantly different than response in rats not given immune TDL, $P > 0{\cdot}5$.

Suppression transferred by spleen cells from s.c. immunized donors. **Effect of LP feeding on transferable mucosal suppression** Spleen cells from s.c. immunized donors are suppressive for a mucosal anti-CT response in adoptive recipients (Koster & Pierce, 1983). To determine whether this phenomenon was altered by protein deprivation prior to donor immunization, spleen cells donors, fed either HP or LP, were immunized s.c. with 40 μg CT or CTd; 4 weeks later spleen cells were harvested and assayed for suppressive activity in non-immune recipients. Table 3 shows that spleen cells from HP fed donors were markedly suppressive in adoptive recipients, jejunal ACC responses being 65–85% lower than in recipients of non-immune spleen cells. In contrast, spleen cells from LP fed donors were suppressive only when LP diet was begun at the time of donor immunization; when LP was started 4 weeks before donor immunization, and continued until cell harvest, no

Table 3. Effect of protein deprivation on suppression transferred by spleen cells from s.c. immunized donors

Spleen cell donors			Spleen cell recipients from LP donors		Spleen cell recipients from HP donors	
Duration of diet (weeks)	Weeks on diet when immunized*	Antigen	Jejunal ACC response†	Percentage suppression‡	Jejunal ACC response†	Percentage suppression‡
8	—	—	13,570 (1·3)	0	11,520 (1·3)	—
4	0	CT	3,960 (1·3)§	66	2,980 (1·6)§	74
8	4	CT	12,080 (1·3)	0	4,000 (1·5)§	65
8	4	CTd	10,900 (1·5)	5	1,770 (1·7)§	85

* Spleen cell donors received 40 μg of CT or CTd s.c. Diet was continued until spleen cells were harvested.

† Non-immune recipients were given $1{\cdot}5 \times 10^8$ spleen cells i.v. and 10 μg CT i.d. The i.d. dose of CT was repeated on day 14 and jejunal biopsies were taken on day 19. Results are expressed as geometric mean ACC × mm^3 ($\overset{\times}{\div}$ s.e.). n = six in each group.

‡ Compared with responses in rats given non-immune spleen cells from HP donors.

§ Significantly lower responses than in rats given non-immune spleen cells from HP donors, $P < 0{\cdot}05$.

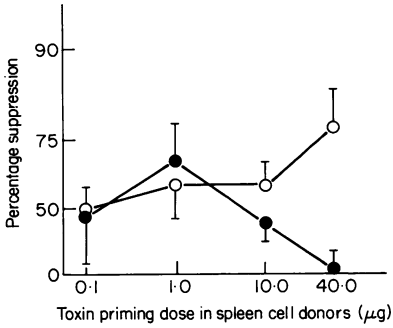

Fig. 1. Effect of s.c. immunizing dose of CT on mucosal suppression transferred by spleen cells from HP (○) and LP (●) fed donors. Spleen cell donors were fed HP or LP for 4 weeks, immunized s.c. with the indicated dose of CT, and continued on the same diet for 4 more weeks until cell harvest. Transferable suppression was studied in recipients (see Materials and Methods) each given spleen cells equivalent to one donor spleen ($0{\cdot}5 \times 10^8$ cells from LP donors, $1{\cdot}5 \times 10^8$ cells from HP donors). Percent suppression was determined by comparison with controls given HP diet and no cells ($11{,}520 \overset{\times}{\div} 1{\cdot}3$). Each point reflects data from six to eight recipients. Results for HP and LP donors are significantly different only for donors immunized with 50 μg CT ($P < 0{\cdot}01$).

suppression was observed. That the effect of LP reflected an antigen specific mechanism is shown by the failure of spleen cells from non-immune donors, fed LP for 8 weeks (or even 16 weeks; data not shown), to be suppressive in adoptive recipients. These results also show that the effect of CT given s.c. to donor rats was due to its antigenicity rather than its toxicity, because non-toxic CTd acted identically.

Effect of s.c. antigen dose on transferable mucosal suppression The suppressive effect of an inoculum of spleen cells depends upon the dose of s.c. antigen given to donors and the interval between immunization and spleen cell harvest (Koster & Pierce, 1983). In further studies, we sought to determine whether the effect of protein deprivation on splenic suppressor cell activity was also influenced by the dose of s.c. antigen given to donors. Spleen cells were collected from donors fed HP of LP for 4 weeks, immunized s.c. with 0·1–40 μg CT, and continued on the diet for 4 more weeks until cell harvest. Transferable suppression was studied in recipients, each given spleen cells equivalent to one donor spleen ($0{\cdot}5 \times 10^8$ cells from LP donors, $1{\cdot}5 \times 10^8$ cells from HP donors). Results in Fig. 1 show that spleen cells from HP fed donors immunized with 0·1–10 μg CT were moderately suppressive in adoptive recipients, and similar results were obtained when spleen cells from LP fed donors were studied. In contrast, spleen cells from HP fed donors immunized with 40 μg CT were highly suppressive, whereas those from identically immunized LP donors were non-suppressive. Although the use of smaller cells inocula from LP fed rats might underestimate their suppressive capacity, these results suggest that LP feeding impairs spleen cell-mediated suppression only when high doses of s.c. antigen, which evoke marked suppressor activity in HP fed donors, are used.

Restoration of transferable suppression by refeeding with HP In the final study we sought to determine whether the diminished suppressive effect of immune spleen cells from LP fed donors could be restored by refeeding the donors with HP after s.c. immunization. Fig. 2 shows that spleen cells from donors fed LP 4 weeks, immunized s.c., and fed LP 4 more weeks before cell harvest (group II) were non-suppressive in adoptive recipients, whereas those from donors fed LP and immunized as above, but also refed with HP for 4 additional weeks before cell harvest (group III), were at least as suppressive as those from donors never given LP (group I). However, when cell donors were fed LP for a total of 12 weeks (8 weeks before s.c. immunization and 4 weeks after) and then refed with HP for 4 weeks (group IV), the suppressive effect of cell transfer was only partly restored; although ACC responses in cell recipients were modestly lower than in controls ($P < 0{\cdot}05$) they were significantly higher ($P < 0{\cdot}05$) than in recipients of cells from donors given LP only 8 weeks before refeeding (group III).

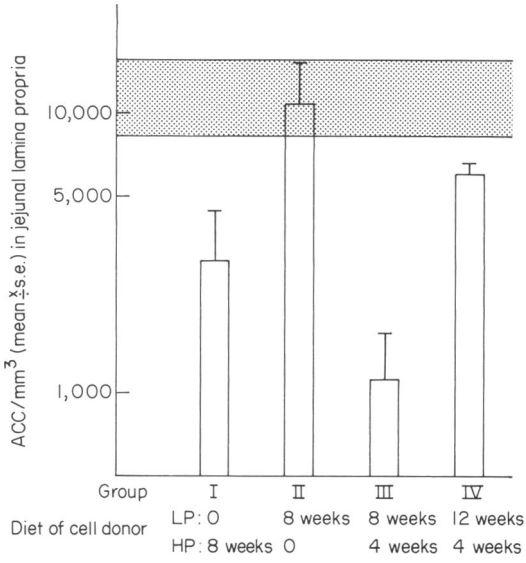

Fig. 2. Restoration of transferable suppression by refeeding of spleen cell donors. Spleen cell donors were fed HP (group I) or LP (groups II & III) for 4 weeks, or LP for 8 weeks (group IV), immunized s.c. with 40 μg CT, and continued on the same diet for 4 weeks; donors in groups III & IV were then refed with HP for 4 weeks. Spleen cells were harvested 4 weeks after immunization in groups III & IV. Transferable suppression was studied in recipients (see Materials and Methods) each given $1{\cdot}5 \times 10^8$ spleen cells i.v. Each mean reflects data from six to 10 recipients. The shaded area indicates the mean ACC response ($\pm$s.e.) in controls given HP diet and no cells. Results in groups I, III & IV differ significantly from those in controls and in group II ($P < 0{\cdot}05$). Results in group III are also significantly lower than in group IV ($P < 0{\cdot}05$).

DISCUSSION

An earlier study has established that protein deprivation impairs the mucosal IgA anti-toxin response to CT in rats (Barry & Pierce, 1979). The purpose of the present studies was to determine the effect of protein deprivation on lymphocyte populations which participate in or modulate mucosal IgA anti-toxin. Lymphocyte populations were studied by an adoptive transfer technique (Pierce & Cray, 1981; Koster & Pierce, 1983), in which cells generated in an immunized, protein deprived host were assayed for function in an intact, normally nourished recipient.

These studies confirm and extend earlier studies on the effect of protein deprivation on mucosal IgA responses. Barry & Pierce (1979) observed that protein deprivation impaired both B immunoblast production in Peyer's patches and antigen driven division of immunoblasts in ileal mucosa. The present studies indicate that protein deprivaion also impairs the development of lymphocytes which adoptively transfer mucosal priming from enterically immunized donors to non-immune recipients. Further studies using allotype congenic rats have shown that transferred immune donor TDL consist of both B cells and T cells (Koster, unpublished observations). Thus it is likely that protein deprivation impairs the development and/or function of T helper cells as well as B immunoblasts.

Protein deprivation severely impaired the production and/or function of suppressor cells in spleen, after s.c. immunization, and in thoracic duct lymph after i.d. immunization. From these observations we conclude that the LP diet impaired both cell populations which amplify, and also those which suppress, the mucosal IgA response. Exactly how these defects contribute to the observed impairment of mucosal IgA anti-toxin responses in intact, protein deprived rats (Barry & Pierce, 1979) is, however, uncertain. Because the net effect of LP feeding was to impair the IgA response, it seems likely that defects in B cell or T helper cell numbers or function were largely responsible.

These studies confirm and extend three previous observations concerning the general effects of protein deprivation on the immune response. First, the ability to show that a response is inhibited by protein deficiency may depend upon the dose of antigen used. We observed that impaired suppressor cell function was seen only at the highest immunizing dose of CT studied. Price (1978) found that protein deprivation impaired priming with aluminum phosphate absorbed tetanus toxoid only at higher doses, whereas after booster immunization the anti-toxin response was impaired only at lower doses of toxoid. The mechanisms underlying these observations are not known.

Second, the degree of impairment of the immune response is determined by the duration of protein deprivation. Thus priming transferred by immune TDL declined when duration of LP feeding prior to immunization increased from 2 to 6 weeks (Table I). Similarly, Barry & Pierce (1979) found that impairment of jejunal ACC responses in intact rats was determined by the duration of LP feeding prior to enteric immunization.

Third, brief periods of refeeding after protein deprivation usually restore immune responsiveness. We observed that refeeding restored transferable priming and suppression, even after 8 weeks of protein deprivation, which is consistent with restoration of the jejunal anti-toxin response in the intact rat (Barry & Pierce, 1979). Refeeding fully rehabitates the immune apparatus with normal growth kinetics (Bell & Hazell, 1977; Malave, Nemeth & Pocino, 1980), and restores tuberculin responsiveness (Sakamoto, Nishioka & Shimada, 1979), despite 8 weeks of protein deprivation. Refeeding also rapidly restores transferable suppressor cell activity (Bongiorni-Malave & Pocino, 1980). In summary, protein deficiency prevents local expansion of antigen triggered precursors, but does not inhibit the antigen sensitization events. Refeeding apparently permits delayed expansion of previously sensitized clones.

Finally, although the LP diet used in this study, and an earlier one (Barry & Pierce, 1979), was designed to create isolated protein deficiency (Hegsted & Chang, 1965), it was also relatively zinc deficient, because no zinc was added. The zinc contained in the dietary casein provided only 0·4–1·7 parts/10^6; however, the amount of zinc obtained from water and the environment is unknown. Thus the LP fed rats, described in this paper as protein deficient, may also have been zinc deficient, and the effects of the LP diet may have been due in part to zinc deficiency. Diets containing 7 parts/10^6 of zinc or less depress growth, antibody production and a number of cell-mediated immune functions (Fernandes *et al.*, 1979). The possibility that zinc deficiency contributed to the present finding is being studied.

This work was supported by Research grant 1 PO1 AI 14480 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health and by a grant from the Rockefeller Foundation. Research facilities were provided by the Gerontology Research Center, National Institute of Aging, National Institutes of Health, under its Guest Scientist Program.

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